

THE EFFECT OF FAST, AND REGENERATION IN LIGHT VS DARK, ON REGULATION IN THE HYDRA-ALGAL SYMBIOSIS¹

PATRICIA BOSSERT AND L. B. SLOBODKIN

Department of Ecology and Evolution, S.U.N.Y., Stony Brook, L.I., New York 11794

ABSTRACT

Green hydra are able to regenerate tentacles after fast durations which cause brown, *i.e.*, asymbiotic, hydra to fail completely, but the presence of endosymbiotic algae does not always enhance regeneration in fasted hydra.

Green hydra whose nutritional state falls below some threshold, exhibit a "light induced" inhibition of regeneration. That is, hydra, fasted in the light, then randomly assigned to light or dark after decapitation, regenerate better in the dark. This effect of light does not appear to be present either in brown hydra or in normally green hydra from which the algae have been removed.

In a large strain of *Chlorohydra viridissima*, after fasts of intermediate duration (10 and 15 days), this light induced inhibition of regeneration is associated with an increase in the number of algae per gastric cell in regenerating hydra relative to non-regenerating controls.

We have not been able to associate this algal increment with an increase in hydra gastric cell mitosis for animals fasted 9 days prior to decapitation and allowed to regenerate 48 hours in the light.

INTRODUCTION

The maintenance of a symbiotic association characterized by long term stability obviously requires regulation of the numbers of one symbiont relative to the other. The mechanism for maintaining such stability will vary with the intimacy of the association, the nature of the exchange or other interactions between the symbionts, and the inherent reproductive capacities of each organism.

In a multicellular organism, when the cell itself becomes the habitat of a symbiont, one may ask: To what extent has the intracellular symbiont become an organelle of the host cell (Margulis, 1970)? This is partially reducible to another question; that is, to what degree is regulation imposed upon the symbiont by the host cell and to what degree does it emanate from other cells (*i.e.*, from the organism as a whole)?

Green hydra, whose gastrodermal cells contain *Chlorella*-like algae, are well suited for studying this question. With a given feeding frequency and light regime, the numbers of algae present are characteristic of both body region (Pardy, 1974) and strain (McAuley, 1979). The reproductive rate of the algae increases about 20 fold when cultured free of the host cell environment (Jolley and Smith, 1978). Under normal conditions the algae are neither digested nor expelled (Muscatine and Poole, 1979).

Regulation at the organismal level was suggested by Pardy and Heacox (1976). Removal of the hypostome and tentacles increased the number of algae in peduncle

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cells. Grafting a hypostome onto the peduncle prevented this algal increase. They suggested that a factor emanating from the hypostome directly affected algal reproduction within the host hydra cell.

Recently, McAuley (1981) repeated the experiment of Pardy and Heacox (1976). He measured changes in algae per cell in regenerating heads (tentacles, hypostome, and a collar of tissue proximal to the hypostome) and peduncles. He found an increase in the number of algae per cell in both tissues suggesting that presence or absence of a hypostome is not the critical factor affecting changes in the number of algae per hydra cell.

Further, he found a higher mitotic index in regenerating peduncle tissue than in non-regenerating controls, 48 hours after cutting. He suggested that the algae increase in number relative to host cells because they complete the mitotic cycle faster than host cells, and that the stimulus for increased mitosis in both host and algal cell is purely a local, cellular phenomenon.

Here we report the preliminary studies which caused us to become interested in the mechanism(s) by which green hydra regulate their endosymbiotic algae.

MATERIALS AND METHODS

Hydra species and maintenance of stock

Hydra used in these studies have been cultured in our laboratory for several years in a controlled temperature chamber at 20°C and in constant light according to the methods of Loomis and Lenhoff (1956). We designate all our green hydra, *Chlorohydra viridissima* (viridis), giving clones of distinct origin a strain name. The larger strain was purchased from Carolina Biological Supply Company (Carolina strain) and the smaller was isolated from the Nissequogue River near Stony Brook, New York (Nissequogue strain). The brown species used were *Hydra americana*, *Hydra oligactis*, and *Hydra cauliculata*. Aposymbiotic clones were prepared from green hydra using Pardy's method (1976).

Since experiments took place over a 5 year period, variation in the frequency with which stocks were fed occurred. However, all animals in a given experiment came from stocks with the same recent feeding history as indicated: Experiment 1 and 2—Stock animals fed 1–3 times per week; Experiment 3 and 4—Stock animals fed Monday through Friday; Experiment 5—Stock animals fed Monday, Wednesday, and Friday.

Hydra stocks are fed, *ad lib* to repletion, with 24–36 hour *Artemia salina* nauplii.

Maintenance and selection of experimental animals

The criteria for selection of animals from various stocks was as follows: Experiment 1—Animals with one small bud on which no tentacles were present; Experiment 2—The largest, non-budding individuals; Experiment 3 and 4—The largest, non-budding individuals that had actually ingested food during the last feeding prior to the onset of fast; Experiment 5—Same as experiment 2.

In all experiments animals were removed from the parent culture, placed in bowls of the same size, and maintained in the same chamber as the parent stock. In the first experiment, three nutritional levels were established as follows: Level 1—Fasted for 5 days; Level 2—Fasted for 5 days, fed 5 hours prior to decapitation; Level 3—Fed daily for 5 days. In the remaining experiments, a fasting experimental stock was established and animals were removed from it periodically for treatment.

Preparation of slides for algal counts

Hydra tissue was macerated in a deep well spot dish or directly on an uncoated glass slide in a drop of macerating fluid (David, 1973). All counts were performed on tissue comprising approximately the upper half of decapitated hydra (*i.e.*, gastric and budding regions) or the middle third of non-regenerating hydra (*i.e.*, gastric and budding regions). In those decapitated hydra where regeneration preceded maceration, newly regenerated tentacles were removed as completely as possible before the assay region was macerated.

Preparation of slides for mitotic assay

Dried gastric tissue macerates were stained for approximately 10 minutes with DAPI (4'6-diamidine-2-phenylindole di-hydrochloride) in 'M' solution (Loomis and Lenhoff, 1956) at a concentration of 1 mg/ml and examined using a Leitz epifluorescent microscope immediately after staining.

Conditions of regeneration

Regeneration occurred in the same chamber as the parent stock (constant light, 20°C). For dark regeneration, dishes containing experimental animals were placed under a cardboard box. Animals regenerating in the dark were exposed to light for a brief period daily while tentacles were counted and measured.

RESULTS

*Effect of fast on degree of regeneration in brown vs green hydra:
Experiment 1, Table I*

Groups of 10 hydra were fed with different frequencies. Animals were decapitated and the number of animals on which tentacles regenerated after 24 and 48 hours in the light was recorded. When fasted for five days green hydra (Nissequogue) regenerated following decapitation but brown hydra (*H. oligactis*, *H. americana*, *H. caudiculata*) did not.

*Effect of fast on degree of regeneration in light vs dark:
Experiment 2, Tables II and III*

Groups of 10 hydra were removed from stock and either decapitated immediately or fasted for 2 days and then decapitated. Five animals of each group were placed in the dark, five remained in the light and both were allowed to regenerate for 96 hours. Animals that disintegrated before 96 hours were discarded from the experiment. Animals that remained intact, but failed to regenerate by 96 hours were scored as zero.

Fed green hydra (Nissequogue) and 2 day fasted green hydra (Carolina) regenerate more tentacles in the dark than in the light. Brown hydra (*H. americana*) regenerate equally well in light and dark (Table II.)

In the last two trials of this experiment, in addition to recording the number of tentacles regenerated, the length:width ratio was also determined for each tentacle in an extended condition. For brown hydra, the length:width ratio of tentacles is identical in light and dark after 96 hours of regeneration. For green hydra, tentacles are longer in dark than in light for fed animals (Nissequogue) and for 2 day fasted animals (Carolina) (Table III).

TABLE I

Effect of fast on regeneration in brown vs green hydra

Feeding level	Hours of regeneration	<i>Hydra oligactis</i>	<i>Hydra americana</i>	<i>Hydra caudiculata</i>	Nissequogue strain
1	24	0	0	0	60
	48	0	0	0	100
2	24	0	0	10	70
	48	0	0	20	100
3	24	60	10	50	90
	48	80	20	60	90

Percent regeneration determined as the number of hydra on which tentacles were visible \div 10 (the total number of hydra in each group).

Changes in the number of algae per gastric cell and degree of regeneration in light vs dark as a function of fast: Experiment 3, Tables IV, V and VI

At intervals of 2, 10, 15, and 20 days, 6 hydra were removed from each fasting stock of Nissequogue and Carolina strains and decapitated. Three animals were placed in dark, three in light and all allowed to regenerate until differences between them appeared. The degree of regeneration was determined for each animal in each treatment. Gastric regions were pooled and macerated as described in Methods. Controls consisted of 3 intact animals fasted for the same number of days and placed

TABLE II

Effect of fast on the number of tentacles regenerated in light vs dark

Strain	Length of fast (days)	Light	Dark	Significance
Nissequogue	0	4.8 \pm 1.4 (25)	5.6 \pm 0.8 (24)	$P > .001$
	2	3.4 \pm 2.8 (11)	4.3 \pm 2.0 (15)	ns
Carolina	0	4.7 \pm 1.8 (25)	4.3 \pm 2.3 (24)	ns
	2	4.9 \pm 0.6 (15)	5.9 \pm 1.1 (15)	$P > .01$
<i>H. americana</i>	0	4.3 \pm 0.9 (19)	3.2 \pm 2.3 (20)	ns
	2	3.2 \pm 2.1 (10)	2.6 \pm 2.4 (10)	ns

Tentacles were counted on groups of five hydra after 4 days of regeneration. Replication of the experiment was as follows: FED—Nissequogue—5; Carolina—5; *H. americana*—4. FASTED—Nissequogue—3; Carolina—3; *H. americana*—2. The number of animals examined is in parentheses. The mean is expressed \pm s.d. The significance of the difference between the means was determined by the Wilcoxon two sample test.

TABLE III

Length:width ratio of tentacles regenerated in light vs dark

Strain	Length of fast (days)	Light	Dark	Significance
Nissequogue	0	1.6 \pm 0.7 (10)	5.3 \pm 0.7 (10)	$P > .001$
	2	2.8 \pm 2.4 (8)	2.8 \pm 2.5 (10)	ns
Carolina	0	3.2 \pm 2.0 (10)	4.7 \pm 2.1 (10)	ns
	2	3.5 \pm 2.5 (10)	8.1 \pm 1.7 (10)	$P > .01$
<i>H. americana</i>	0	1.5 \pm 0.7 (10)	0.9 \pm 0.9 (10)	ns
	2	1.6 \pm 1.0 (10)	1.0 \pm 0.8 (10)	ns

Tentacles were measured in an extended condition on groups of 5 hydra after 4 days of regeneration. The mean is expressed \pm s.d. The number of hydra examined is in parentheses. The significance of the difference between the means was determined by the Wilcoxon two sample test.

in either light or dark for the same time as the regenerating animals. Gastric regions from these animals were excised, pooled and macerated as above.

Degree of regeneration in light vs dark

For Carolina fasted 10, 15, and 20 days the degree of regeneration was greater in the dark. That is, Carolina regenerates marginally more ($P = .1$) and longer (.05 $> P > .025$) tentacles in dark than in light after fasts of 10, 15, and 20 days. For the smaller Nissequogue strain, those animals surviving similar conditions appeared to regenerate equally well in light and dark (Table IV).

Number of algae per gastric cell in animals regenerating in light vs dark

For Nissequogue, the only significant increase in the number of algae per gastric cell, is in animals regenerating in the light after 15 days of fast prior to decapitation

TABLE IV

Number, and length:width ratio of tentacles regenerated in light vs dark in fasted green hydra

		Light	Dark	Significance
Nissequogue	Tent/Polyp	3.1 \pm 2.3	2.3 \pm 2.4	ns
	L:W	2.1 \pm 3.2	0.86 \pm 0.90	ns
Carolina	Tent/Polyp	3.0 \pm 1.7	4.2 \pm 1.1	$P = .1$
	L:W	1.1 \pm 0.63	3.1 \pm 1.7	.05 $> P > .025$

The results of fast durations 10, 15, and 20 days are pooled. Thus, for Nissequogue in light $n = 10$; dark, $n = 7$ and for Carolina in light $n = 10$; dark, $n = 11$. The means are expressed \pm s.d. Significance of the difference between means was determined by the Wilcoxon two sample test.

(Table V). This algal increase is not associated with a diminished capacity to regenerate in the light, however (Table IV). For Carolina, there is a significant increase in the number of algae per cell after both 10 and 15 days of fast for animals regenerating in the light (Table V). These same animals show a significantly lower degree of regeneration in the light (Table IV).

Degree of regeneration per polyp and number of algae per cell is shown in Table VI for Carolina. When fast durations of 10, 15, and 20 days precede decapitation, Carolina regenerates more and longer tentacles in the dark than in the light. For 10 and 15 days of fast, this light induced inhibition of regeneration is associated with a significant increase in the number of algae per gastric cell in decapitated animals relative to non-decapitated controls. For 20 days of fast, a light induced inhibition of regeneration is clear, but it is not associated with an increase in the number of algae per cell in the regenerating animals.

Effect of fast on degree of regeneration in light vs dark in aposymbiotic hydra: Experiment 4, Table VII

At intervals of 2, 10, 15, and 20 days, 6 hydra were removed from fasted aposymbiotic stocks of Carolina and Nissequogue strains and decapitated. Three animals were placed in dark, three in light, and all allowed to regenerate for 72 hours. (After 15 days of fast, Nissequogue animals appeared about to disintegrate. The degree of

TABLE V

Number of algae per gastric cell in hydra regenerating in light vs dark

Day of fast	Hours of regeneration	Strain	Light		Dark	
			Decapitated	Control	Decapitated	Control
2	72	C	15.1 \pm 9.6 (3)	14.4 \pm 5.8 (3)	16.6 \pm 6.8 (2)	17.5 \pm 7.6 (3)
		N	16.2 \pm 6.8 (3)	16.0 \pm 8.0 (3)	15.4 \pm 9.6 (3)	16.0 \pm 7.4 (3)
10	48	C	17.7 \pm 8.0** (1)	14.6 \pm 10.0** (3)	20.7 \pm 9.3 (3)	21.2 \pm 10.9 (3)
		N	23.9 \pm 9.1 (3)	25.5 \pm 11.4 (3)	20.7 \pm 9.6 (3)	25.0 \pm 11.1 (3)
15	96	C	30.2 \pm 11.7*** (3)	21.1 \pm 9.3*** (3)	21.0 \pm 9.5 (3)	— (0)
		N	26.9 \pm 13.1** (3)	21.2 \pm 10.8** (1)	22.0 \pm 9.0 (1)	22.8 \pm 8.0 (1)
20	72	C	20.6 \pm 9.0 (3)	21.7 \pm 10.1 (3)	16.2 \pm 9.5 (3)	22.4 \pm 11.3 (3)
		N	—	—	—	—

* C = Carolina; N = Nissequogue.

The mean is expressed \pm s.d. The number of hydra from which cell samples were pooled is in parentheses. For each sample $n = 100$ cells. Since the distribution of algal count data is not normally distributed, a t test was performed on log transformed data (Sokal and Rohlf, 1969).

TABLE VI

Regeneration and number of algae per gastric cell in hydra regenerating in light vs dark after a fast

Fast (days)	Regeneration				Number of algae per cell			
	Tent./Polyp		Length:width		Light		Dark	
	L	D	L	D	Dec.	Control	Dec.	Control
	—	3	—	1				
10	—	3	—	1	17.7 ± 8.0	14.6 ± 10.0	20.7 ± 9.3	21.2 ± 10.4
	0	3	0	1				
15	4	5	1	5				
	4	5	2	3	30.2 ± 11.7	21.1 ± 9.3	21.0 ± 9.5	—
	5	5	1	3				
20	2	6	1	5				
	4	4	2	4	20.6 ± 9.0	21.7 ± 0.1	16.2 ± 9.5	22.4 ± 11.3
	2	4	2	5				

The number of tentacles regenerated and the length:width ratio is presented for each of three experimental animals. A (—) indicates that an animal died before tentacles regenerated. Algal counts were performed on 100 gastric cells pooled from animals surviving the fast. A (—) indicates algal count data was lost.

regeneration in both strains was therefore recorded at 48 hours for these 15 day fasted animals.)

Data in Table VII shows that fasted, aposymbiotic hydra of either strain do not regenerate better in the dark. This is in contrast to what we found for fasted green hydra for which light is sometimes detrimental to regeneration.

*Mitotic index of gastric cells in fasted hydra regenerating in light:
Experiment 5, Table VIII*

After 9 days, 28 animals of fasted Carolina stock were decapitated, and allowed to regenerate for 48 hours in light. The gastric regions were excised, pooled 7 to a slide, and macerated as described in Methods. Controls were gastric regions from intact hydra fasted for 11 days.

Before determining the mitotix index in fasted, regenerating hydra, we tested the DAPI staining technique developed by Muscatine and Neckelmann (1981).

TABLE VII

Regeneration in light vs dark in fasted aposymbiotic hydra

		Light	Dark
Nissequogue	Tentacle/Polyp	2.9 ± 2.4	3.3 ± 2.2
	Length:Width	3.9 ± 6.9	3.5 ± 3.9
Carolina	Tentacle/Polyp	3.6 ± 1.7	2.7 ± 1.5
	Length:Width	4.3 ± 3.4	1.0 ± 0.77

The results are pooled for 2, 10, 15, and 20 days of fast. For Nissequogue regenerating in the light $n = 10$; in the dark, $n = 11$. For Carolina regenerating in the light, $n = 12$; in the dark, $n = 11$. The means are expressed ± s.d.

TABLE VIII

Mitotic index and number of algae per gastric cell in hydra regenerating after fast

Strain	Control		Regenerating	
	Mitotic index	Algae/cell	Mitotic index	Algae/cell
Carolina	0.4	24.2 \pm 9.9	0.4	25.4 \pm 8.1
	0.4	23.0 \pm 8.2	0.2	25.0 \pm 9.8
	0	22.6 \pm 11.1	0	27.6 \pm 11.2
	0.4	23.6 \pm 10.6	0	25.6 \pm 11.7
Mean (n = 4)	0.3 \pm 0.2	23.4 \pm 0.7*	0.15 \pm 0.7	25.9 \pm 1.2*

Each of the replicates represents a slide on which the gastric regions from 7 hydra were pooled and macerated. The number of algae per cell was determined by counting algae present in 60 gastric cells. The mitotic index was determined by counting the number of mitotic figures in 500 gastric cells. The significance of the difference between the number of algae per cell in regenerating hydra vs controls was determined by *t* test on untransformed data, *df* = 3 (Sokal & Rohlf, 1969).

Under conditions designed to give maximum mitosis (10 hours after feeding) they report a mitotic index of 1.22 ± 0.16 s.e. for fed hydra and 0.54 ± 0.13 s.e. for unfed controls in the Florida strain. We repeated their procedures, except that animals were assayed 12 hours after feeding and we used Nissequogue strain. Finally, mitotic figures were identified and counted on slides without the observer knowing if she was examining fed hydra or controls. We found a mitotic index of $0.9 \pm .1$ s.e. in fed hydra compared to $0.4 \pm .1$ s.e. for controls.

Satisfied with our use of the technique, we proceeded to apply it to determine changes in the mitotic index and number of algae per cell in fasted, regenerating hydra. In Carolina strain fasted for 9 days and regenerating 48 hours in the light, the number of algae per gastric cell is significantly higher than in controls ($P = .05$). We found no increase in the mitotic index of gastric cells in these same animals.

DISCUSSION

The light-induced inhibition of regeneration is not associated with any absolute length of fast. Rather, it appears to occur under conditions where the nutritional state of the green hydra falls below some critical level *i.e.*, in 0 and 2 day fasted animals removed from stocks fed erratically (1–3 times/week) and after 10–20 days of fast in animals removed from heavily fed stocks. It is not clear if there is a strain specificity associated with this response. Under those conditions where Carolina clearly regenerates better in the dark (after fasts of 15 and 20 days: Table VI), individual Nissequogue polyps frequently failed to regenerate.

When algal endosymbionts are removed from hydra, the detrimental effect of light on regeneration is also removed (Table VII). There is some indication that light-induced inhibition of regeneration is associated with an increase in the number of algae per cell in the regenerating hydra, at least for the larger strain, Carolina (Table VI). For the smaller, Nissequogue strain, an algal increase in regenerating hydra is found only after 15 days of fast and no light induced inhibition of regeneration is associated with it (Table V).

Fasting green hydra show a net accumulation of glycogen and a gradual catabolism of protein (Cook and Kelty, 1982). There is a significant "back transfer" of metabolites from hydra to algae (Thorington and Margulis, 1981). If this "back

transfer" is light driven, and if any of these metabolites are rate limiting with respect to regeneration, then a reasonable mechanism for light-induced inhibition of regeneration exists.

McAuley (1981) demonstrated that algal mitosis occurs under conditions where the host hydra cells are stimulated to divide. For example, he found that the mitotic index of regenerating peduncle cells is higher than non-regenerating controls. He suggests that the transient increase in algal cells relative to hydra cells, observed in regenerating peduncles, occurs simply because algal cells divide faster than host cells. Thus, when hydra tissue suffers a wound, both host and symbiont are stimulated to divide. Algal cells complete the mitotic cycle faster than host cells and a transient increase in their numbers within the hydra cells occurs.

In contrast, McAuley (1981) found no increase in the number of algae per cell in gastric tissue when hydra regenerated after a 1 day fast prior to decapitation. Similarly, we found no increase in the number of algae within gastric cells in hydra regenerating after a fast of 2 days, when hydra were taken from heavily fed stocks (Table V).

We could interpret our findings in terms of McAuley's hypothesis as follows: In well-fed hydra the number of cells in the gastric region is sufficient to regenerate missing parts solely by migration of cells. Therefore, since no hydra cell mitosis occurs prior to regeneration (Hicklin *et al.*, 1973; Webster, 1967; Corff and Burnett, 1969) algal cells are apparently not free to divide, and no transient increase in their numbers occurs.

After a fast of 10 to 15 days we found that the number of algae per gastric cell does increase, however, in regenerating hydra. We hypothesized that if an algal increase is observed, we would also find host cell mitosis elevated in regenerating animals. We did not. In Carolina, after a 9 day fast, the number of algae per gastric cell in regenerating animals is higher than non-regenerating controls after 48 hours of regeneration, but the mitotic index in these same animals is not higher than controls (Table VIII).

This result caused us to re-examine the proposal that a substance emanating from the hypostome can by itself directly regulate algal mitosis (Pardy *et al.*, 1976). Thus, when the hypostome is removed, an increase in algal cells relative to host cells could occur without a concurrent increase in host cell mitosis. It is not clear why this effect of decapitation on algal numbers is found only in fasted hydra, however. It is possible that one of the effects of fast is simply to alter the "timing" of algal mitosis with respect to mitosis of the host cell. Prolonged fast may selectively delay the animal cell's ability to respond to a mitotic stimulus. Under such conditions algal numbers could be elevated 48 hours after a wound without a concurrent increase in host cell mitosis.

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